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Differentiation of pluripotent stem cells into endothelial cells

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Abstract

Purpose of Review—Methods to isolate endothelial cells from murine and human pluripotent stem cells continue to evolve and increasingly diverse endothelial cell populations have been generated. This review provides an update of key papers published within the past year that report on some of those advances.

Recent findings—Cooperative interactions among microRNA (miRNA), transcription factors, and some downstream interacting proteins have been reported to enhance endothelial specification from embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). Endothelial cell differentiation can also be modulated by various growth factor additions, Notch pathway activation or inhibition, and modulation of the microenvironment of the differentiating ESC and iPSC. Functionality of the derived endothelium has been demonstrated by a variety of *in vitro* and *in vivo* assays. Finally two recent reports have identified endothelial progenitor populations with robust proliferative potential.

Summary—Progress in differentiating endothelial cells from ESC and iPSC has been made. The recent report of formation of endothelial colony forming cells from human ESC and iPSC provides a protocol that can generate clinically relevant numbers of cells for human cell therapy.

Keywords

Embryonic stem cells; Induced pluripotent stem cells; Endothelial cells; Endothelial colony forming cells

Introduction

Endothelial cells (EC) line the blood vessels that are diversified from simple capillaries formed by single EC into large multi-layered and multi-cellular conduit vessels in the arterial, venous, and lymphatic systems. A greater appreciation of the tissue heterogeneity of EC within specific tissues and organs has been achieved through some seminal publications that highlight specific angiocrine factors in modulating tissue functions [1–3]. As the transcriptional profiles and cell and tissue specific patterns of gene expression regulating

Conflicts of interest

None

cell function become more clarified, targeted profiles for tissue specific EC phenotypes have become of interest for scientists generating EC from murine and human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). Several recent reviews have highlighted potential heterogeneities in the EC derived from ESC and iPSC [4–7]. This review will focus on some recent advances in generating EC from murine and human ESC and iPSC published within the past 12 months.

Studies with murine ESC or iPSC

In general, EC are derived from differentiating ESC and iPSC via 3 primary methods [7]. First, ESC and iPSC can be differentiated in conditions that promote self-aggregation of the cells into three dimensional embryoid bodies (EB) in a suspension culture. Numerous lineages of cells will emerge within the EB which spontaneously undergo differentiation into the 3 germ layers: ectoderm, endoderm, and mesoderm. It is from the mesoderm subset that both hematopoietic and EC emerge. The EC self-organize into vascular structures within the EB. Addition of a variety of growth factors will promote enhanced EC differentiation within the EB. In a second method, differentiating ESC and iPSC are co-cultured with some type of stromal cell to promote EC lineage differentiation from the mesoderm. Murine calvarial mesenchymal cells called OP9 cells are widely used to promote differentiation and facilitate emergence of cardiac, hematopoietic, endothelial, and other lineages. Finally, some investigators prefer differentiating the ESC and iPSC in 2 dimension culture on tissue culture plates coated with protein substrates (Matrigel, fibronectin, gelatin, or other proteins) using specific culture mediums with sequentially added recombinant growth factors. No matter which of the 3 methods are chosen, numerous lineages of cells emerge from the proliferating and differentiating ESC and iPSC and use of growth factors, growth factor inhibitors, small molecules, and neutralizing antibodies can be used to promote enrichment of the lineage of choice. In general, use of monoclonal antibodies to identify specific cell surface molecules or genetic tagging of the ESC or iPSC with lineage specific fluorescent reporter systems are required for each of the 3 methods of differentiation to identify the cell lineage of choice and to enrich the cells using some form of cell sorting and isolation [7]. Key steps in the emergence of the murine EC are the early expression of Flk1 in Brachyury (BRY) expressing mesoderm cells that requires activing A/nodal signaling. Activation of bone morphogenetic protein 4 (BMP4) signaling further enhances formation of the mesoderm subset giving rise to blood and EC. Subsequent addition of numerous growth factors such as vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and placental growth factor (PlGF) all further promote EC proliferation, differentiation and eventual maturation during the in vitro culture.

While most reports have identified the mesoderm germ layer as the source of endothelial precursors, Goldman et al. [8] have recently presented evidence that some EC derived from differentiated murine and human ESC are derived from the endoderm lineage. Using established protocols for deriving enriched endoderm precursors in ESC-derived EBs, hepatic precursors were identified and characterized and certain subsets were shown to give rise to EC. Subsequent examination of human fetal liver specimens (7 – 7.5 week-old fetal samples) provided confirmation that some liver EC co-express endothelial and hepatic

markers. A variety of in vitro and in vivo vascular functional analyses confirmed that the endoderm-derived EC display mature endothelial functions. Validation of the role of endoderm as a source for some liver EC was provided via the use of dual-reporter transgenic mice ESC differentiation cultures and use of a mouse endoderm lineage-tracing model that confirmed marking of fetal liver hepatoblasts and some liver EC. Whether or not these liver EC are restricted to formation of sinusoidal endothelial cells remains to be determined, but these findings suggest one potential mechanism to generate tissue specific EC.

Some important transcription factors that promote EC emergence from mesoderm precursors have been identified recently. Cha et al. [9] have reported that an isoform of the transcription elongation factor TFIIS called *Tcea3*, is highly enriched in mouse ESC and negatively regulates Activin A/Nodal signaling. When *Tcea3* transcripts were diminished using a shRNA plasmid in murine ESC, the ESC displayed enhanced expression of mesoderm markers including BRY, VEGFA, and VEGFC with loss of expression of pluripotiency gene transcripts. Consistent with enhanced mesoderm formation, greater numbers of EC with enhanced tube formation were observed when the murine ESC with diminished *Tcea3* transcripts were cultured in vitro. These studies suggest that *Tcea3* plays an important role in EC differentiation from murine ESC via modulation of Activan A/Nodal signaling.

Certain transcription factors appear to be critical for differentiation of EC from murine ESC. *Er71/Etv2*, a member of the E-twenty six (ETS) transcription factor family has been reported to be necessary for mesoderm specification giving rise to both blood and EC lineages during murine, zebrafish, and *Xenopus* development [10,11]. *Er71/Etv2* is known to directly bind to numerous genes that are critical for blood and EC lineage development including *Flk1*, vascular endothelial-cadherin *(VE-cad)*, stem cell leukemia *(Scl)*, and limb domain only 2 (*Lmo2*). Kim et al. [12] have recently identified a transcription factor Ovo-like zinc finger 2 (OVOL2), a C2H2 zinc finger protein, as a novel interacting protein for *Er71/Etv2*. They reported that OVOL2 and ER71 co-localized in the nucleus of hematopoietic cells upon immunohistochemical staining with antibodies. While OVOL2 alone failed to activate the *Flk1* promoter, it significantly enhanced ER71-mediated *Flk1* promoter activation. Co-expression of ER71 and OVOL2 in differentiating murine ESC led to a significant increase in endothelial and hematopoietic cell production. When *Ovol2* transcripts were diminished using a short-hairpin RNA-mediated inhibition approach, significant loss of ESC differentiation into blood and EC was observed. They concluded that OVOL2 is an interacting transcription factor that directly binds to ER71 and is critical for the differentiation of blood and EC from murine ESC.

Shi et al. [13] have reported that the GATA2 transcription factor is co-expressed and directly interacts with ER71 in EC and hematopoietic cells of murine embryos. Increased co-expression of GATA2 significantly augments ER71 in the production of endothelial and blood cells from differentiating murine ESC. Evidence is presented that ER71 and GATA2 directly bind to the *Spi1* transcription factor gene both in vitro and in vivo in early murine embryogenesis and this pathway may be critical for regulating mesoderm differentiation into endothelial and hematopoietic cells.

Vereide et al. [14] performed a screening of various candidate molecules known to be important for hematopoietic differentiation via use of a doxycycline inducible cassettes in murine ESC, fetal liver cells, and/or fibroblast cells. Six transcription factors including *Gata2*, *Lmo2*, *Mycn*, *Pitx2*, *Sox17*, and *Tal1* were demonstrated to impose a program in transfected cells that restricted them in a proliferative state with hemangioblastic potential that could be released upon removal of the doxycycline induction and EC, blood cells, and some smooth muscle cells emerged. A central role for FGF2 in stimulating the "expandable hemangioblasts" to proliferate more efficiently and to demonstrate greater migratory behavior with a flatter morphology (all evidence of a less differentiated state). The authors conclude that these results imply that it is possible to identify and control the state of various lineage progenitor cells and this ability may permit identification of the molecules required to trap, expand, and study progenitors in lineages currently poorly maintained in culture.

EC lineage emergence from differentiated iPSC may also be regulated by specific micro-RNA (miR). Di Bernardini et al. [15] reported that miR-21 was one of the most overrepresented miR expressed as murine ESC were differentiated into EC. When miR-21 was overexpressed in differentiating ESC, numerous endothelial lineage markers were increased along with increases in capillary tube-like structures in vitro and greater vascularization in Matrigel plugs implanted in vivo. The authors confirmed a role for activation of the Akt signaling pathway in mediating the miR-21 induced effects to promote endothelial differentiation. Chen et al. [16] reported that miR-199 is increased in a step-wise fashion as murine ESC differentiate into EC. MiR-199 was observed to target and suppress expression of the Notch ligand Jagged 1 (JAG1) and this resulted in an increase in VEGF transcription and secretion in a STAT3 dependent fashion. Use of short-hairpin RNA-mediated knockdown of JAG1 expression ablated the effect of miR-199 on EC differentiation. Murine ESC-derived EC transfected with miR-199 demonstrated increased capillary tube-like formation in vitro and in vivo vascularization in implanted Matrigel plugs. Of interest, overexpression of miR-199 inhibited differentiation of vascular smooth muscle cells from differentiating ESC and led the authors to conclude that miR-199 may serve as a regulator of the phenotypic switch controlling EC versus smooth muscle cell differentiation.

As an example of the use of iPSC differentiation to serve as a tool for understanding a clinical condition, Gu et al. [17] examined whether iPSC-derived EC derived from subjects with diet-induced obesity (DIO) would exhibit signs of endothelial dysfunction (as is known to occur in the obese subjects) or would be reprogrammed and not display endothelial dysfunction. The iPSC-derived EC generated from DIO mice displayed significantly less migratory activity, proliferation, and decreased capillary tube-like formation in vitro; all signs of EC dysfunction. DIO iPSC-derived EC injected into the muscle of animals that had undergone acute ischemic injury induction failed to improve reperfusion compared to wild-type iPSC-derived EC. Of interest, co-infusion of pravastatin into the site of hindlimb ischemia along with the DIO iPSC-derived EC led to a significant improvement in reperfusion in a nitric oxide dependent fashion. The authors concluded that this is the first evidence that DIO iPSC-derived EC display endothelial dysfunction and raises questions

about use of iPSC derivation from obese human subjects for generating cell therapies to treat EC dysfunction in these subjects.

Studies with human ESC or iPSC

Culture methods to differentiate EC from human ESC or iPSC generally follow the 3 methods described above. However, recent advances in some culture methods have provided for more enriched populations of EC or combinations of desired cell lineages with EC to promote tissue recovery in various animal model systems. Zhang et al. (Zhang S et al. Biomaterials 2014) reported that human iPSC can be plated as a single cell suspension in a 3D fibrin scaffold with up to 45% of the differentiated iPSC displaying EC markers at day 14 of culture. Upon purification, more than 95% of cultured cells displayed EC markers for at least one month. The authors conclude that this method for deriving EC is highly efficient. The exact ratio of iPSC:EC was not reported for this protocol and the overall number of EC produced during the one month culture was not discussed; two important variables when contemplating development of protocols for generating cells for human cell therapy.

Masumoto et al. [18] developed a culture system to simultaneously generate cardiomyocytes, EC, and vascular mural cells from human iPSC over a 18 day period. The heterogenous differentiated population of cells is then cultured on temperature-responsive adhesive culture plates that permit development of tissue sheets that can be overlaid to produce cardiac tissue-like multi-layered structures. Transplantation of the “cardiac tissue sheets” into hearts of athymic nude rats that had undergone experimental sub-acute myocardial infarction led to significant improvements in cardiac function with engraftment of human cardiomyocytes in >40% of the transplanted animals. The authors conclude this protocol may be useful to apply for human cardiac regenerative therapy. Skelton et al. [19] developed a cell surface marker detection system to define cell lineage relationships during cardiomyocyte differentiation from human iPSC. Specific cell surface markers were identified that permitted isolation and discrimination of EC from cardiac lineage specified cells. It is proposed that use of the identified markers will permit a starting approach to map early human cardiogenesis.

Orlova et al. [20] developed an iPSC differentiation protocol for the simultaneous derivation of EC and pericytes. On day 10 of iPSC differentiation, from 10–30% of cells displayed CD31 and/or CD34 expression (EC markers) while 30% expressed platelet derived growth factor receptor beta (mesenchymal marker). The EC and pericytes displayed stable cell surface markers and lineage functions; for example, human iPSC-derived EC engrafted in zebrafish embryos and integrated into the embryonic zebrafish vasculature. Park et al. [21] also developed a iPSC-derived EB system for generating cells that displayed EC/pericyte markers they termed vascular progenitor (VP) cells. The VP cells developed were capable of engrafting into the injured retinal capillaries of mice that had been subjected to high intraocular pressure to induce retinal ischemia/reperfusion injury and persisted up to 45 days in vivo. Surprisingly, the EC generated from iPSC derived from cord blood CD34 expressing cells were superior to EC generated from iPSC derived from fibroblast cells or EC generated from ESC in homing, engrafting, and persisting in the injured retina.

Several recent publications have begun to more closely assess the functions of EC derived from iPSC or ESC to primary EC populations in human subjects. Reed et al. [22] reported that human umbilical vein EC (HUVEC), blood outgrowth EC (BOEC), and iPSC-derived EC all displayed both toll-like receptor-4 (TLR4) and nucleotide-binding oligomerization domain-containing protein-1 (NOD1) molecules that are critical for sensing and binding gram negative bacterial organisms. In contrast, ESC-derived EC fail to express TRL4, but do bind and respond to gram negative bacteria via expressed NOD1. These data suggest that ESC-derived EC may escape unwanted TRL4 mediated inflammatory situations and this may represent a potential advantage over other EC sources. Reed et al. [23] also reported that HUVEC, BOEC, human aortic EC (HAEC), and human lung microvascular EC (HMVEC) all secreted high and comparable levels of the vasoconstrictor endothelin-1 and the cardio protective hormone prostacyclin; ESC-derived EC failed to express either of these molecules. Culture of the EC under shear stress caused the BOEC and HAEC to elongate and align to the direction of the shear stress, while ESC-derived EC failed to respond to shear stress with any changes in morphology. These results suggest that BOEC (derived as circulating cells in the peripheral blood) are more similar to HUVEC, HAEC, and HMVEC than the ESC-derived EC. Since other groups have reported that ESC-derived EC may respond to shear stress with changes in morphology [24,25], these results also raise the question as to whether the EC derived from ESC may be heterogenous in their functional properties depending on the ESC line or conditions of differentiation.

Sahara et al. [26] have recently reported derivation of an endothelial progenitor (EP) population of cells displaying expression of CD31, CD34, and KDR, but not CD14 in a multi-step 14 day differentiation protocol from an endothelial specific VE-cad promoter reporter ESC line. The iPSC:EC ratio of 1:20 was reported to be a significant improvement over prior publications. The EP population (identified on day 6–7 of the protocol after 2 differentiation steps) outperformed the more mature EC generated during step 3 of the differentiation protocol (days 7–14) with respect to the number of blood vessels formed in implanted Matrigel plugs and the EP persisted in the host immunodeficient mice for 3 months without tumor formation. The authors conclude that the ESC-derived EP are more readily produced and display superior vessel forming ability compared to more mature EC and may be an attractive cell population for revascularization strategies in human subjects.

Prasain et al. [27] report the most significant advance for the field of scientists interested in generating EC from human ESC or iPSC. They have developed a novel protocol that relies upon identification of a Neuropilin-1 (NRP-1) expressing subset of EC co-expressing CD31 and CD144 that display functional properties similar to umbilical cord blood endothelial colony forming cells (UCB-ECFC) with high clonal proliferative potential and robust in vivo vessel-forming ability. The ESC-NRP-1^{+}CD31^{+}ECFC and iPSC-NRP-1^{+}CD31^{+}ECFC maintained a stable endothelial phenotype and function and did not undergo replicative senescence for 18 passages in vitro. Given the high rate of proliferation, the iPSC:EC ratio was identified as 1:1 × 10^{8}; by far the most robust generation of functional EC that has been reported. These EC engrafted and formed functional human vessels in immunodeficient mice for > 6 months. Furthermore, the iPSC-NRP-1^{+}CD31^{+}ECFC rescued the avascular regions and blunted the neovascularization observed in vehicle treated mice that had undergone oxygen-induced retinopathy induction. Finally, the iPSC-NRP-1^{+}CD31^{+}ECFC

significantly rescued blood flow to the ischemic hindlimb of mice that had undergone femoral excision to a degree similar to UCB-ECFC infusion. These data indicate that there is a unique population of ESC-NRP-1^{+}CD31^{+}ECFC and iPSC-NRP-1^{+}CD31^{+}ECFC that can be identified using specific differentiation conditions that can provide clinically relevant numbers of highly functional EC that may be a useful cell therapy to employ in patients that display vascular dysfunction.

Conclusion

Numerous advances in the derivation, expansion, and functional analysis of EC derived from ESC and iPSC have been made in the past year. This overview has identified some of those recent advances. It is apparent that it is now feasible to generate clinically relevant numbers of EC from ESC and iPSC to consider for use as a cell therapy in human subjects.

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Key points

- Endothelial cells can be derived from murine and human ESC and iPSC
- ESC- and iPSC-derived endothelial cells display many features of primary endothelial cells
- A novel protocol to generate clinically relevant numbers of endothelial cells from human ESC and iPSC has been reported